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Latent Essential Fatty Acid Deficiency in a Special Diet Deteriorates Skin Barrier

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Abstract

Background

Recently, a new atopic dermatitis (AD) mouse model was reported; after feeding hairless mice with a special low magnesium diet (HR-AD), they developed dry skin (low skin water content and high transepidermal water loss (TEWL)) and elevated blood immunoglobulin E levels (IgE) with 100% repeatability. Therefore, this model was used for therapeutic research regarding AD. However, to determine the mechanism by which HR-AD induces AD-like symptoms, the relationships between low magnesium, essential fatty acids (EFA), and lipid composition need to be clarified.

Methods

First, to clarify whether the low magnesium of HR-AD induces AD-like symptoms, hairless mice were fed HR-AD or magnesium-supplemented HR-AD. They were evaluated based on TEWL values, an *in vivo* water sorption-desorption test, water intake, serum IgE levels, histological examination, and macroscopic observation. Second, to investigate whether the EFA deficiency in HR-AD induces AD-like symptoms, hairless mice that had previously developed AD-like symptoms were fed olive oil-soaked HR-AD. Finally, the lipid composition of HR-AD was analyzed by gas chromatography.

Results

Comparing with the HR-AD group revealed that magnesium supplementation did not compensate for any skin symptoms. However, feeding with olive oil-soaked HR-AD for 3 days dramatically cured the AD-like symptoms. On re-feeding with HR-AD, the AD-like symptoms were recovered. Lipid analysis revealed that HR-AD did not contain linoleic and linolenic acids.

Conclusions

We clarified that latent EFA deficiency but not magnesium deficiency induces AD-like symptoms in the new AD mouse model following HR-AD feeding.

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Key Words: Atopic dermatitis; Animal model; Essential fatty acids; Transepidermal water loss

Introduction

Atopic dermatitis (AD) is a chronically relapsing skin disease that occurs most commonly during early infancy and childhood. The clinical features of AD are characterized by uncontrollable pruritus of the skin that induces scratching behavior and results in the development of dermatitis. AD is frequently associated with elevated immunoglobulin E (IgE) levels, a personal or familial history of AD, and allergic rhinitis and/or asthma. Although AD is known to be influenced by both internal factors (inherent skin barrier dysfunction and excessive IgE production against antigens) and external factors (e.g., mechanical irritation and environmental allergens)[1], its pathogenesis remains obscure.

For therapeutic research regarding AD, many animal models have been proposed; they comprise naturally occurring models, hapten-induced models, and Th2 cytokine overexpression models[2-5]. Each model has human AD-like properties; however, the models also have disadvantages in reproducibility or productivity or availability. Thus, new animal models for AD are desired. The diet-induced dermatitis model is one such model. It is known that diets that are very low in magnesium induce skin rashes, severe pruritus, and an increase in serum IgE levels in rats[6]. Since AD-like symptoms are easily induced in this model, it has been used for therapeutic research regarding AD[7]. However, these diets also decrease body weight, which is not a characteristic of AD and are not widely available.

A new diet that is moderately low in magnesium (HR-AD) was reported to induce AD-like symptoms and increase the water intake without weight reduction[8,9]. Since in this model, AD-like symptoms are induced quickly, HR-AD has been frequently used to study the efficacy of drugs and health foods for AD[10-13]. However, the causative mechanism has not been well documented.

Aside from magnesium deficiency, deterioration of the skin barrier is a hallmark of essential fatty acid (EFA) deficiency[14,15]. The adequacy of this model cannot be estimated without nutrition studies, in particular EFA deficiency. The aim of this study was to elucidate the role of magnesium and EFA deficiencies in the induction of AD-like symptoms by HR-AD.

Materials and Methods

Three experiments were performed. First, in order to clarify whether the low magnesium content of HR-AD induced AD-like symptoms, we used magnesium-supplemented HR-AD that contained as much magnesium as the control diet. Second, to investigate whether EFA deficiency was responsible for the AD-like symptoms in this model, we used olive oil-supplemented HR-AD. Finally, we analyzed the lipid composition of HR-AD.

Animals

Female hairless mice (HR-1) aged 3 weeks were obtained from Hoshino Experimental Animal Center (Yashio, Japan). All the mice were fed a standard diet for 1 week and randomly divided into 2 or 3 groups in magnesium supplement experiment and olive oil supplement experiment, respectively. Die and water are available *ad libitum*. According to the feeding protocols, the diets were fed *ad libitum*.

Feeding experiments were performed under specific pathogen-free environments at constant temperature (22-24℃) and humidity (45-50%). They conformed to the Guidelines for Animal Experiments of Osaka City University.

Diets

The low magnesium (100 mg/kg) diet (HR-AD: Lot, E0124) was purchased from Nosan Corp. (Yokohama, Japan); it contains fat (0.3%), protein (21.4%), fiber (4.4%), ash (5.6%), and moisture (2.1%). The control mice were fed a standard diet (MF: Lot. 001004, Oriental Yeast Co. Ltd., Chiba, Japan) that contained magnesium (2700 mg/kg), fat (5.5%), protein (23.7%), fiber (2.9%), ash (6.1%), and moisture (7.3%).

For the magnesium supplement experiment, HR-AD supplemented with as much magnesium (2800 mg/kg) as in the standard diet purchased from Oriental Yeast Co. Ltd. was used. For the olive oil supplement experiment, we soaked HR-AD in olive oil (Lot.V9H2150, Nacalai Tesque, Inc. Kyoto, Japan) overnight to prepare EFA-enriched HR-AD. The olive oil contained oleic (77.3%), palmitic (10.4%), linoleic (7.0%), stearic (3.1%), palmitoleic (0.7%), a-linolenic (0.6%), arachidic (0.4%), icosenoic (0.3%), and behenic (0.1%) acids; it contained no minerals.

Feeding Protocols

1. Magnesium supplement experiment

The mice were divided into 3 groups: HR-AD, magnesium-supplemented HR-AD, and standard diet (control) for 41 days.

2. Olive oil supplement experiment

First, all mice were fed HR-AD for 35 days. After AD-like symptoms had developed in all mice, 1 group was fed olive oil-soaked HR-AD for 4 days. After being fed with a diet supplemented with olive oil, this group continued to be fed HR-AD for 45 days (a total of 84 days). The other group was maintained on HR-AD feeding. The duration of olive oil feeding was determined based on the clinical changes in the mice.

Measurement of skin barrier function

1. Transepidermal water loss

Transepidermal water loss (TEWL) was measured on the dorsal skin twice a week with a Tewameter TM 300 (Courage+Khazaka electronic, Koln, Germany), as previously reported[16].

2. *In vivo* water sorption-desorption test

The *in vivo* water sorption-desorption test was performed once a week with a Skicon-200 hygrometer (I.B.S. Co., Ltd., Hamamatsu, Japan), according to the procedure described by Tagami et al[17]. Briefly, after a drop of water was used to humidify the dorsal skin for 10 seconds, the conductance was measured at an interval of 30 seconds for 2 minutes. The time-series value of the conductance was plotted on graph paper, and it produced a line. The area under the line curve calculated using the trapezoidal method was defined as the water-holding capacity of the stratum corneum.

Quantification of body weight and drinking water

The weights of the mice and drinking water bottles were measured twice a week. Average water intake was calculated based on the decrease in the water weight.

Serum IgE level

Mouse blood was collected under anesthesia at the end of the magnesium supplement

experiment and centrifuged. Serum IgE levels were measured with the IgE enzyme immunoassay kit (Yamasa Corp., Tokyo Japan). The IgE values were used for statistical analysis after logarithmic transformation.

Histological examination

Mice were killed humanely at the end of the magnesium supplement experiment. In the olive oil supplement experiment, 1 mouse from both groups was randomly euthanized to obtain skin samples on day 42 since the commencement of the experiment and day 7 since the olive oil supplementation. Skin samples, which were obtained from the back, were stained with hematoxylin and eosin and examined.

Lipid composition analysis

The composition of fatty acids (oleic, linoleic, palmitic, and linolenic acids) in HR-AD was analyzed using gas chromatography at the Japan Food Research Laboratories (Tokyo Japan). The manufacturer's specifications were referred to for the composition of fatty acids in the standard diet.

Statistical analysis

Statistical analysis of TEWL, water-holding capacity, and serum IgE levels was performed using two way repeated measure ANOVA and Bonferroni/Dunn post hoc test in Magnesium supplement experiment with StatView-J 5.0 statistical software (SAS Institute, Cary, NC, USA). In olive oil supplement experiment, two way repeated measure ANOVA and the Student's t-test was used for statistical analysis. $p<0.01$ was considered statistically significant.

Results

Magnesium supplement experiment

A total of 14 mice were used for this experiment; 4 mice were fed a standard diet (control); 5, the special diet (HR-AD); and 5, magnesium-supplemented HR-AD.

1. Effect of HR-AD and magnesium-supplemented HR-AD on TEWL and water-holding capacity of the stratum corneum

The TEWL values of the mice fed HR-AD exceeded those of the controls from day 18 onwards and increased significantly ($p<0.01$) from day 21 till the end of the experiment. However, they did not significantly differ from those of the magnesium-supplemented group mice (Fig. 1). The water-holding capacity of the HR-AD group mice decreased significantly ($p<0.01$) from day 21 till the end of the experiment. However, it was not significantly different from that of the magnesium-supplemented group mice (Fig. 2).

2. Effect of HR-AD and magnesium-supplemented HR-AD feeding on body weights and water intake

Body weights of the HR-AD group mice tended to be lower than those of the controls; however, the difference was not significant (data not shown). The water intake of the HR-AD group mice exceeded that of the controls from day 18 till the end of the experiment. However, it was not significantly different from that of the magnesium-supplemented group mice (Fig. 3).

3. Effect of HR-AD and magnesium-supplemented HR-AD on serum IgE levels

The IgE levels of the HR-AD group mice significantly exceeded those of the controls ($p<0.01$) (Fig. 4). No significant differences were observed between the serum IgE levels of the HR-AD and magnesium-supplemented HR-AD group mice.

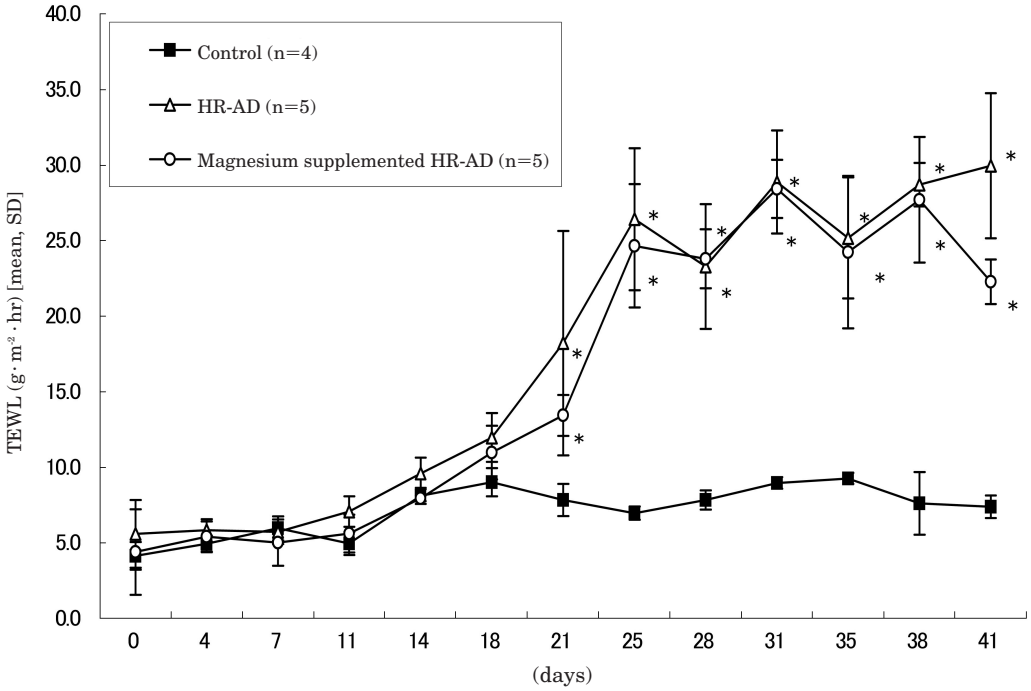

Figure 1. Transepidermal water losses (TEWL) of magnesium supplemented group and HR-AD group increase and significantly exceed one of control during the time course. TEWL was measured on the dorsal skin with Tewameter TM300 at a constant temperature (22-24 degree Celsius) and a constant humidity (45-50 percent). *$p<0.01$ using two way repeated measure ANOVA and Bonferroni/Dunn post hoc test.

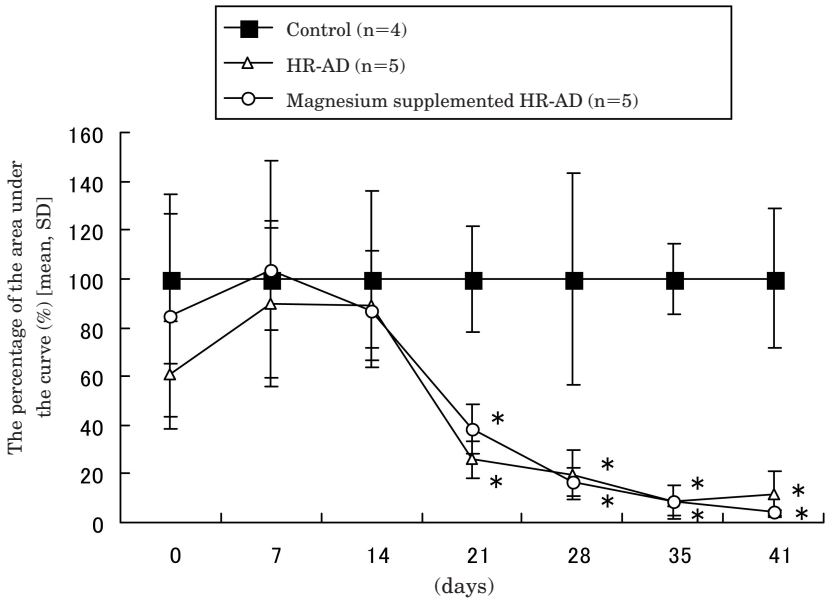

Figure 2. Corneal water holding capacity of magnesium supplemented group decreases as HR-AD group during the time course. They significantly drop below the water holding capacity of control group. Water holding capacity of stratum corneum was measured by in vivo water sorption-desorption test, using a Skicon-200 hygrometer. Values given are the mean SD. *$p<0.01$ using two way repeated measure ANOVA and Bonferroni/Dunn post hoc test.

4. Effect of HR-AD and magnesium-supplemented HR-AD on macroscopic and microscopic findings

The HR-AD and magnesium-supplemented group mice developed skin dryness and wrinkle-

like changes from day 28 till the end of the experiment (Fig. 5B and 5C). On the other hand, the control group mice did not develop AD-like skin changes throughout the entire experimental period (Fig. 5A). The stratum corneum and epidermis of the HR-AD and magnesium-supplemented HR-AD group mice were obviously thickened (Fig. 6).

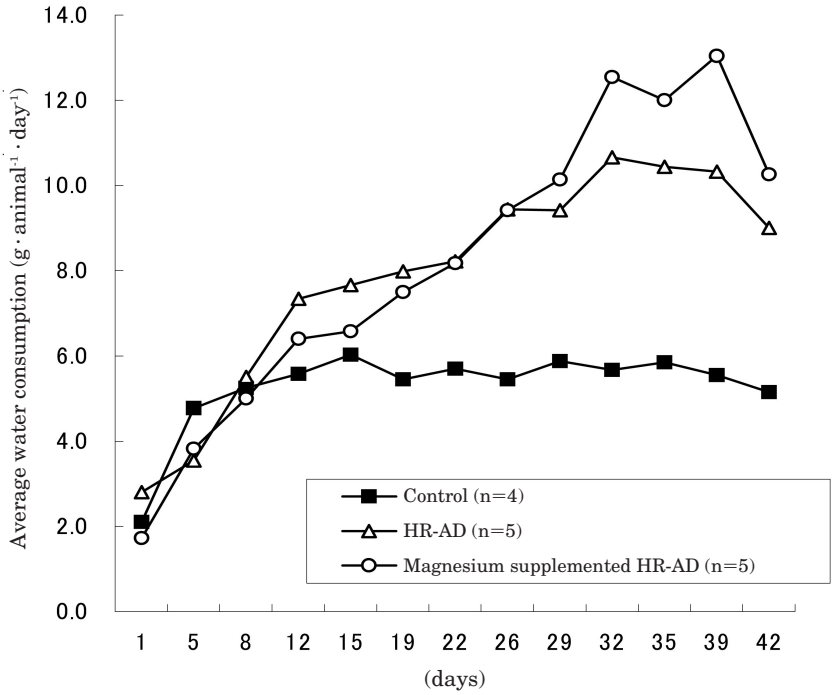

Figure 3. The amount of water intake of magnesium supplemented group increases as much as HR-AD group during the time courses. Mice were fed standard diet (control) containing magnesium 2700 mg/kg or HR-AD diet containing magnesium 100 mg/kg or magnesium supplemented HR-AD containing magnesium 2800 mg/kg. Average water intake was calculated from diminution of water bottle weight.

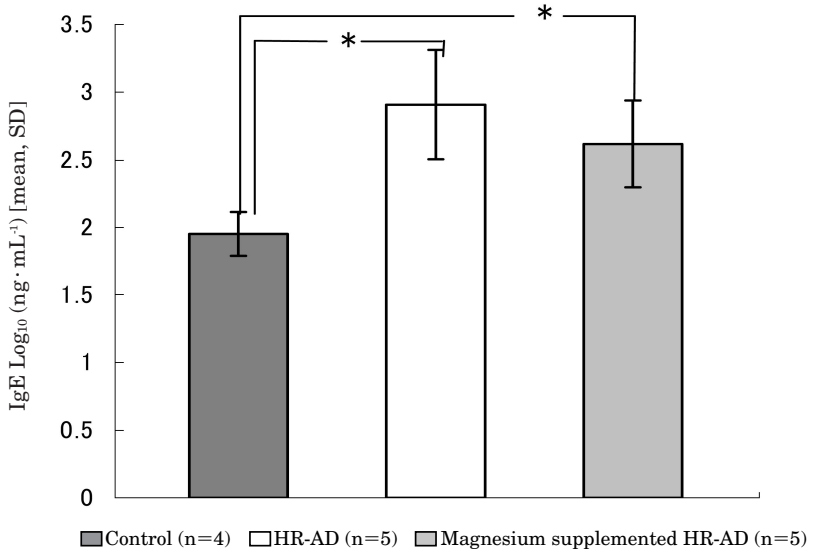

Figure 4. Serum IgE levels of magnesium supplemented group increases as HR-AD group. They significantly increase compared with control. Serum samples are taken at the end of the magnesium supplement experiment and measured with IgE enzyme immunoassay kit (Yamasa Corp., Tokyo Japan). Values given are the mean SD. *$p<0.01$ using Bonferroni/Dunn post hoc test.

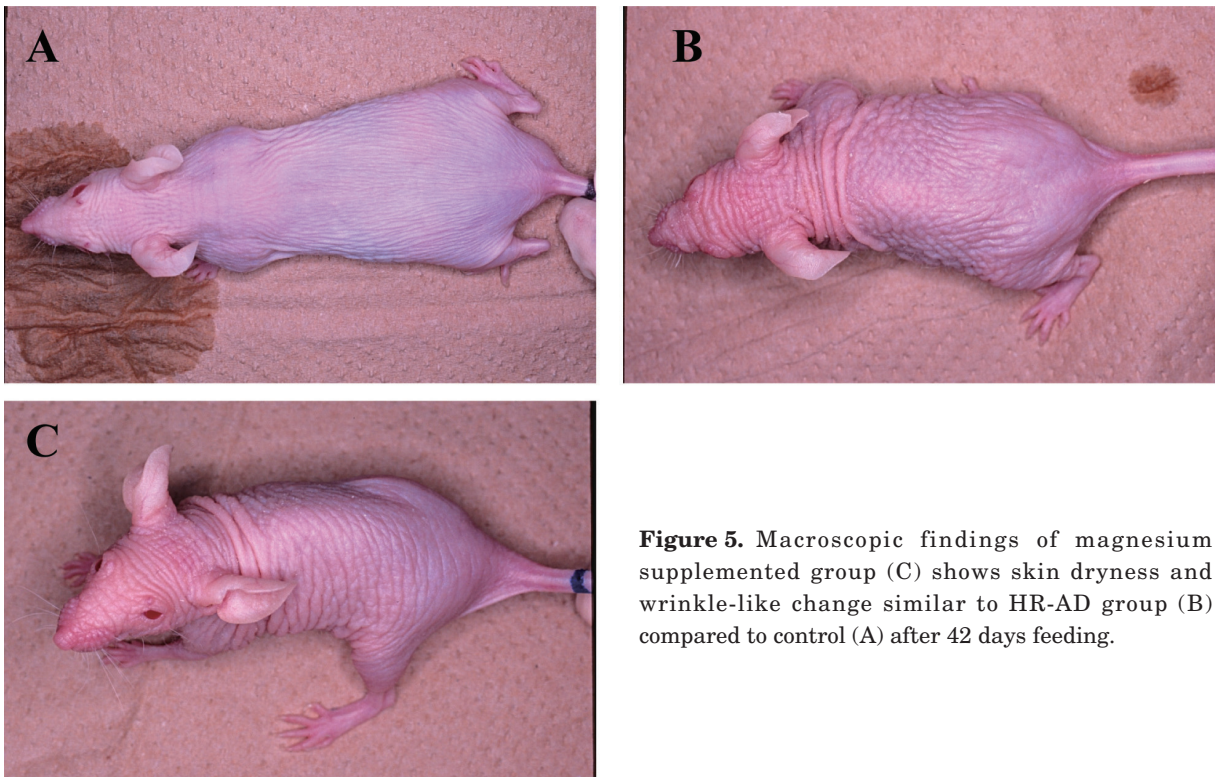

Figure 5. Macroscopic findings of magnesium supplemented group (C) shows skin dryness and wrinkle-like change similar to HR-AD group (B) compared to control (A) after 42 days feeding.

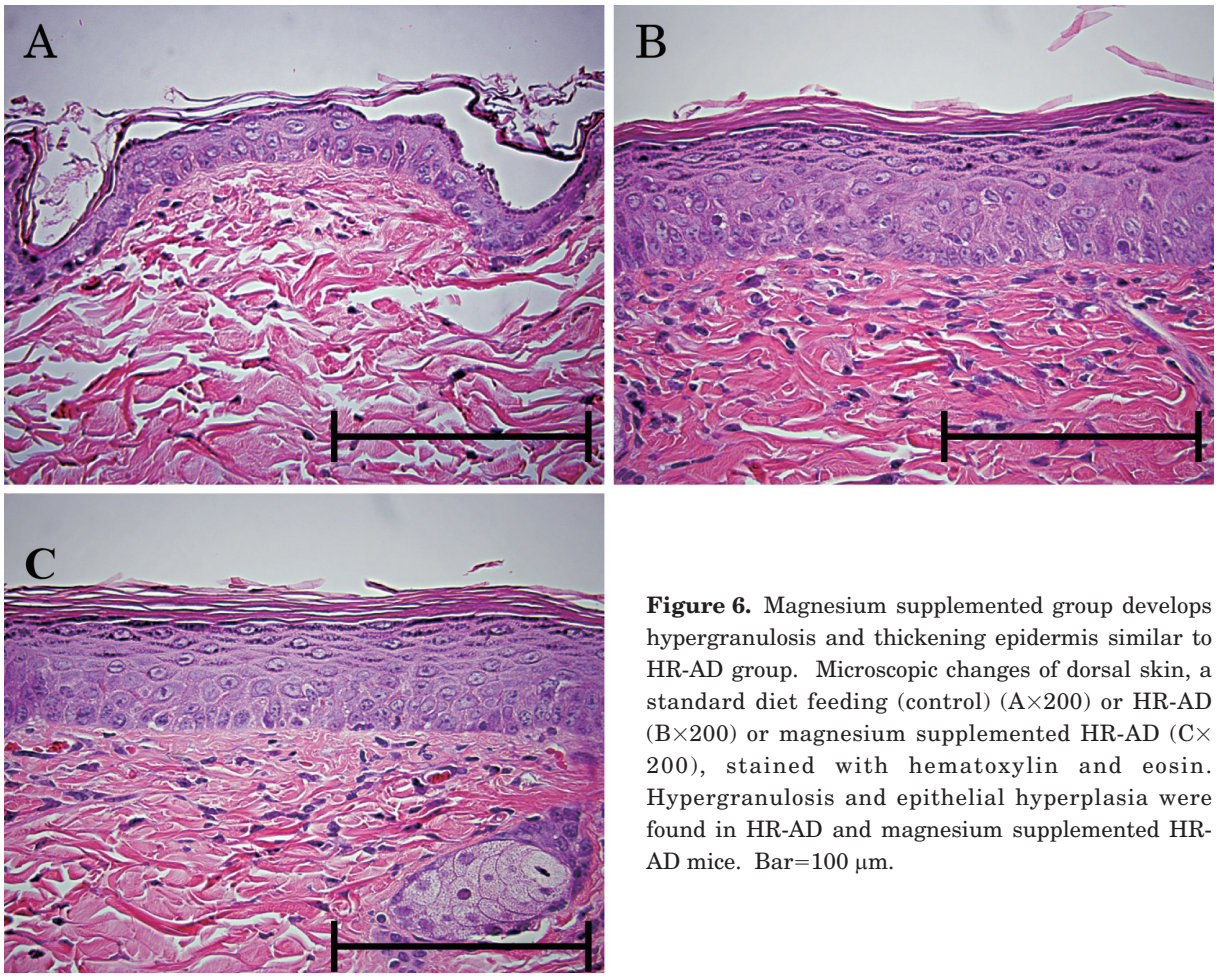

Figure 6. Magnesium supplemented group develops hypergranulosis and thickening epidermis similar to HR-AD group. Microscopic changes of dorsal skin, a standard diet feeding (control) (A×200) or HR-AD (B×200) or magnesium supplemented HR-AD (C×200), stained with hematoxylin and eosin. Hypergranulosis and epithelial hyperplasia were found in HR-AD and magnesium supplemented HR-AD mice. Bar=100 μm.

Olive oil supplement experiment

A total of 11 mice were used for the EFA supplement experiment. Of these, 5 received HR-AD, and 6, olive oil-supplemented HR-AD. Mice that were fed olive oil lived longer than those fed HR-AD. All the mice fed HR-AD died within 61 days (Fig. 7). On the other hand, the olive oil-supplemented group survived for at least 91 days (42 days after consuming olive oil).

1. Effect of olive oil supplementation on TEWL

The TEWL promptly decreased after 4 days of olive oil supplementation (Fig. 8). The TEWL values of the olive oil-supplemented group mice decreased significantly ($p<0.01$) from days 39 to 54. Moreover, from day 50 till the end of the experiment, these values increased again.

2. Effect of olive oil supplementation on body weights and water intake

From days 46 to 54, the body weights of the olive oil-supplemented group mice increased significantly as compared to those of the HR-AD group mice ($p<0.01$) (data not shown). The water intake of each group exhibited the same trend as that of the TEWL (data not shown).

3. Effect of olive oil supplementation on macroscopic and microscopic findings

HR-AD feeding for 35 days induced skin dryness, wrinkle-like changes, and scratching behavior in all the mice (Fig. 9A). Remarkable remission of AD-like symptoms was observed after olive oil supplementation (Fig. 9B). However, it recurred from day 68 onward (29 days after olive oil supplementation) (Fig. 9C).

On day 42, the HR-AD-fed mice showed hypergranulosis and epithelial hyperplasia on microscopic examination of the skin (Fig. 10A). On the other hand, the olive oil-supplemented mice did not show these changes (Fig. 10B). Histological findings of the olive oil-supplemented mice closely resembled those of the controls that were fed the standard diet during the magnesium supplement experiment.

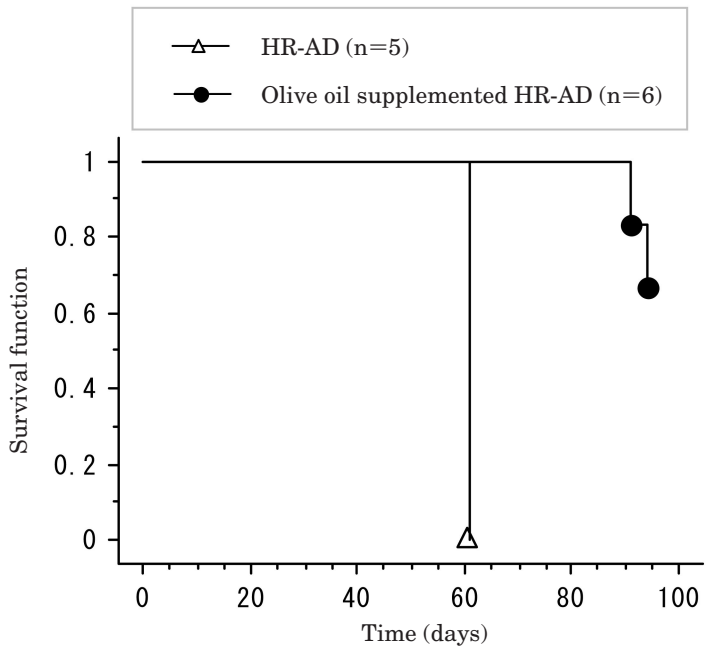

Figure 7. Kaplan-Meier survival curves of olive oil supplement experiment, plotted with StatView-J 5.0 statistical software (SAS Institute, Cary, NC, USA). All HR-AD fed mice died after 61 days. After 91 days, one olive oil supplemented mouse died. After 94 days, two mice of olive oil supplemented group died.

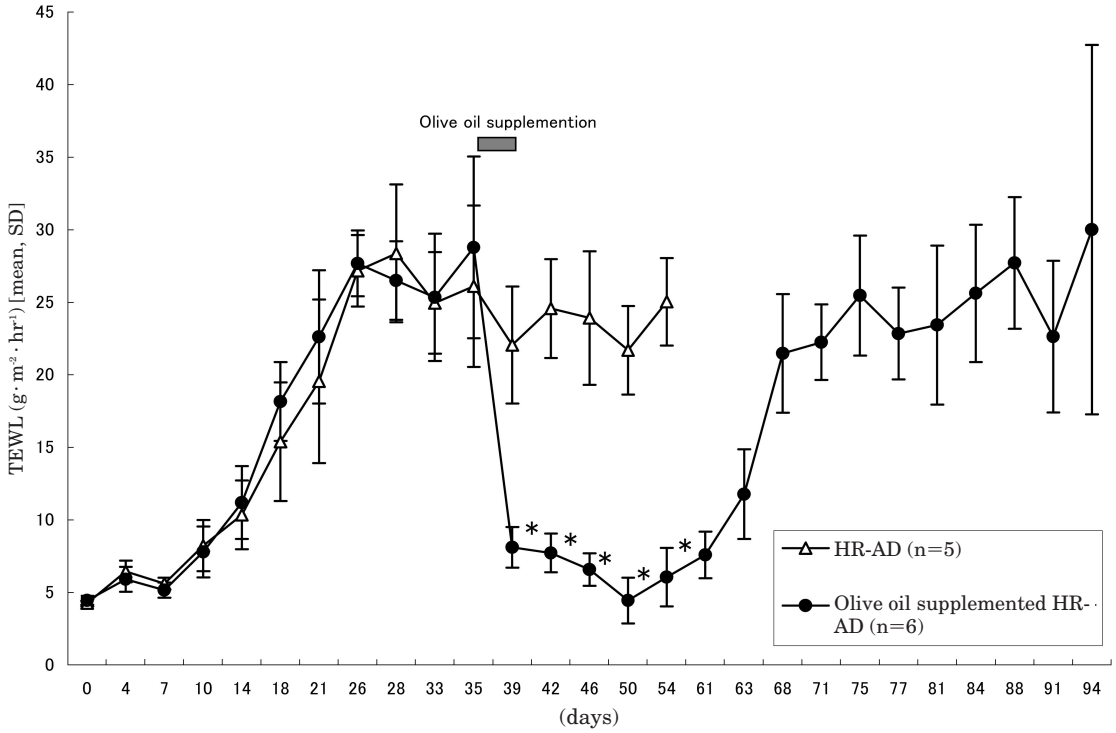

Figure 8. Olive oil supplementation prevents increasing of TEWL caused by HR-AD diet. After olive oil supplementation, TEWL of olive oil supplemented group drastically decreased. After 61 days (13 days after olive oil supplementation), TEWL of olive oil supplemented group increased again. At first, all mice were fed HR-AD for 34 days. From 35 to 39 days, one group fed olive oil supplemented HR-AD. From 40 days after to the end of experiment, olive oil supplemented group was fed HR-AD again. Values given are the mean SD. *$p<0.01$ using two way repeated measure ANOVA and the Student's t-test.

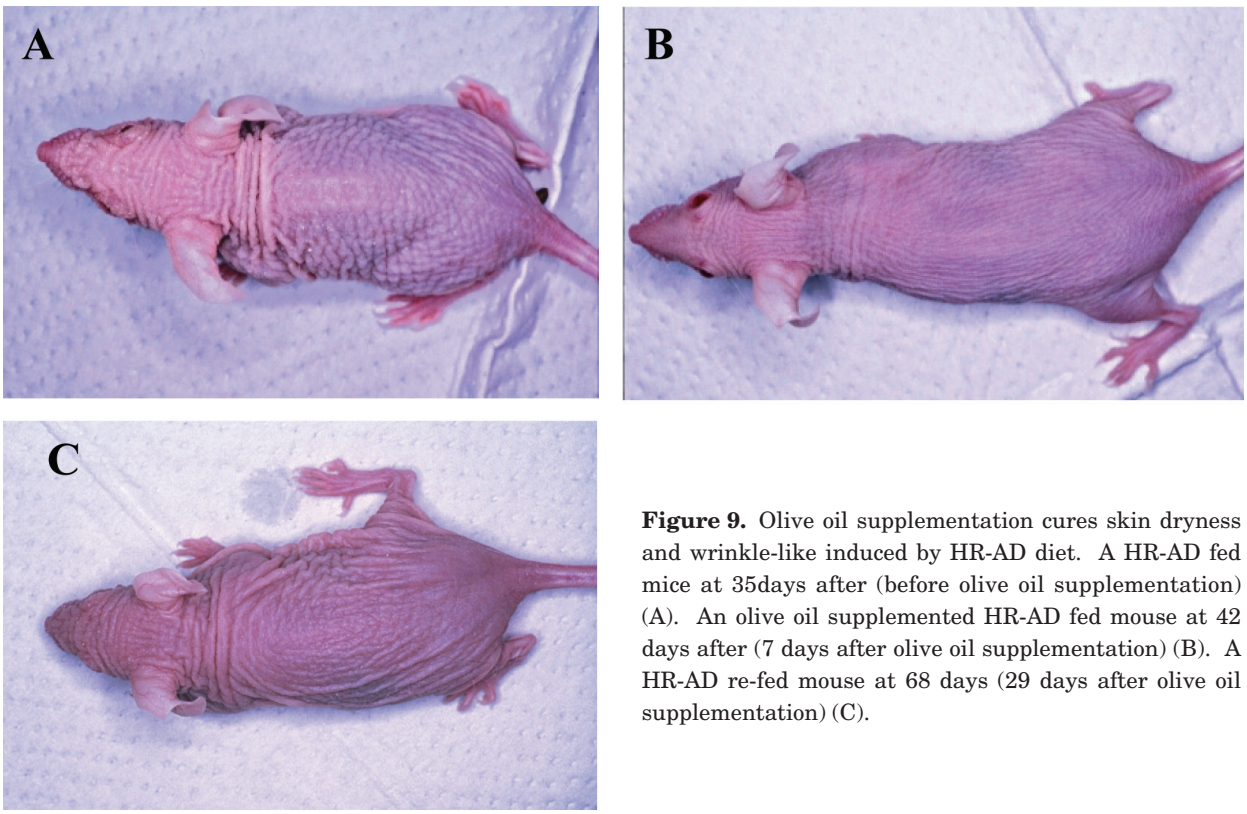

Figure 9. Olive oil supplementation cures skin dryness and wrinkle-like induced by HR-AD diet. A HR-AD fed mice at 35days after (before olive oil supplementation) (A). An olive oil supplemented HR-AD fed mouse at 42 days after (7 days after olive oil supplementation) (B). A HR-AD re-fed mouse at 68 days (29 days after olive oil supplementation) (C).

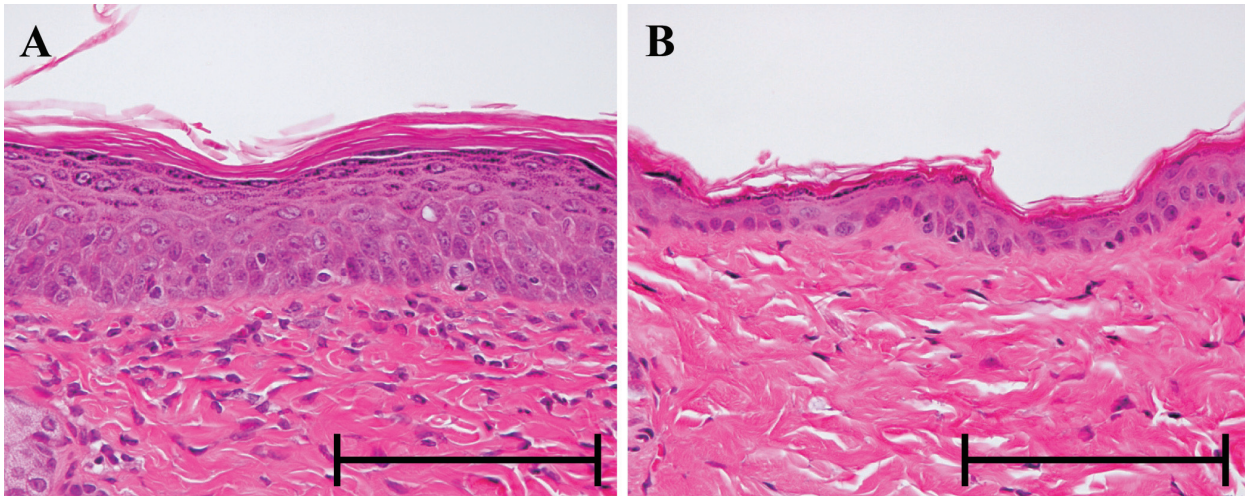

Figure 10. Olive oil supplementation inhibits hypergranulosis and thickening epidermis caused by HR-AD diet feeding. Microscopic findings of dorsal skin, HR-AD fed mice (A×200) and olive oil supplemented HR-AD fed mice (B×200) at 42 days (7 days after olive oil supplementation). Hypergranulosis and epithelial hyperplasia disappeared in olive oil supplemented mice. Bar=100 μm.

Lipid composition analysis

Lipid composition analysis did not detect linoleic and linolenic acids in HR-AD but detected palmitic (0.06%) and oleic acids (0.05%). In contrast, the standard food contained linoleic (2.39%), linolenic (0.19%), palmitic (0.75%), and oleic (1.36%) acids (Table 1).

Table 1. lipid composition of diets (% by weight)

diet	linoleic acid (%)	linolenic acid (%)	palmitic acid (%)	oleic acid (%)
Control diet (MF)	2.39	0.19	0.75	1.30
HR-AD	ND	ND	0.06	0.05

ND: not detectable.

Discussion

Although magnesium deficiency had been thought to be responsible for the AD-like symptoms in this model[8], magnesium supplementation did not inhibit the development of AD-like symptoms (Figs. 1-6). This agreed with the report of Fujii and suggested that certain factors of HR-AD other than magnesium deficiency induced the AD-like symptoms[9]. Neckermann et al reported that low magnesium (12 mg/kg) diets induced acrodermatitis-like symptoms such as reddening and swelling of the ear lobes in hairless rats[7]. However, neither our study nor other researches that used HR-AD have reported similar changes[8-13].

Results of the olive oil supplement experiment revealed that olive oil dramatically cured AD-like physiological and morphologic abnormalities in this model (Figs. 7-9). Furthermore, lipid composition analysis revealed that HR-AD is an EFA-deficient diet (Table. 1). Consequently, these results indicate that EFA deficiency is the major factor of HR-AD that induces AD-like symptoms. Fujii et al described that feeding HR-AD with EFA, such as linolenic acid and linolenic acid, inhibit dry skin symptoms in their preliminary experiment[9]. Whereas they

unpublished their observation and did not show how assess dry skin symptom and why they tried to EFA supplementation, not knowing about EFA deficiency of HR-AD. Our result complements their assumption that EFA deficiency should mainly contribute to the induction of dry skin in HR-AD mice.

Deterioration of the skin barrier is a hallmark of EFA deficiency. In a paper that established the concept of EFA, Barr and Barr reported that fat-deficient diets induced xerosis and that increasing the water intake of rats and administering linoleic acid cured them[18]. EFA deficiency increases the water intake and TEWL in rats without increasing urine production[19]. Lowe and Stoughton histologically demonstrated that EFA-deficient diets induced diffused thickening of the skin, acanthosis, hypergranulosis, and hyperkeratosis in hairless mice[20]. The AD-like symptoms of the HR-AD-fed mice are consistent with these reports.

EFA deficiency depresses the skin barrier function due to structural changes in ceramides. Elias and Brown reported that abnormal barrier function in EFA-deficient mice is associated with the decreased elaboration and deposition of epidermal intercellular lipids[21]. EFA deficiency empties lamellar granules and disrupts the regularity of the intercellular lipid layer in the stratum corneum[22]. Subsequent studies confirmed that EFA deficiency replaces linoleic ester, which plays a crucial role in ceramide-1 structure, with oleic ester in the amide linkage of omega-acylceramides[23,24]. The structural change in ceramide increases the TEWL and decreases the water-holding capacity. These evidences support our conclusion that HR-AD causes deterioration of the skin barrier function due to EFA deficiency.

Although many reports have clarified the features of EFA-deficient animals, little has been reported regarding the increase in serum IgE levels. Bibel DJ et al reported that EFA-deficient mice supported 100-fold more bacteria on their skin, mainly comprising *Streptococcus aureus*, than normal mice[25]. *S. aureus* proteins and toxins are reported to upregulate IgE synthesis in AD[26,27]. On the other hand, ceramides are known to seal intercellular spaces and to stop invading antigens. Irregular lamellae of intercellular lipids caused by structural changes in ceramides increase the permeability of the skin barrier[21]. These evidences support the view that hypofunction of the skin barrier, which occurs in EFA deficiency, enhances the permeability to antigens and leads to an increase in the number of antigens, thereby inducing excess production of serum IgE.

In conclusion, this study clarified that latent EFA deficiency but not magnesium deficiency induces AD-like symptoms in a new AD mouse model obtained by HR-AD feeding. This model is a potential new skin barrier-deterioration model. At the same time, the symptoms in this model were improved by EFA administration. We believe that it is necessary to review other reports that use this model, in view of EFA deficiency.

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